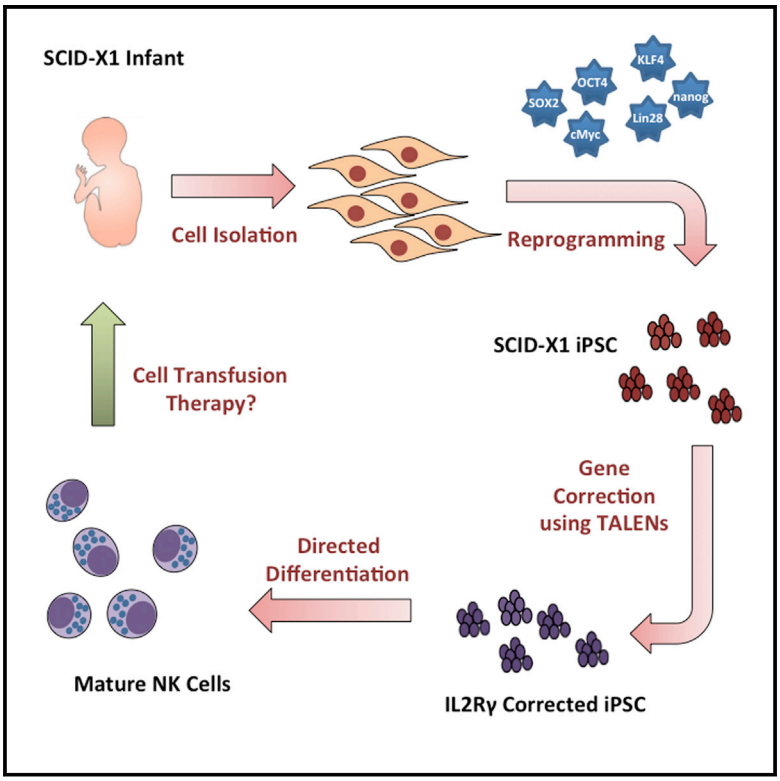
Brief Report

Cell Stem Cell

Lymphoid Regeneration from Gene-Corrected SCID-X1 Subject-Derived iPSCs

Graphical Abstract



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In Brief

Menon, Firth, and colleagues show that TALEN-mediated correction of a novel splice site mutation in the IL-2Rγ gene rescues defective development of mature NK cells from iPSCs derived from a SCID-X1 subject. They demonstrate correction of the aberrant splicing of the IL-2Rγ in T cell precursors.

Highlights

- A splice site mutation in IL-2Rγ was corrected at the endogenous locus using TALENs
- Hematopoietic precursors and myeloid cells develop as normal from SCID-X1 iPSCs
- Mature NK cells were generated from ESCs and iPSCs
- Lymphoid differentiation recovered from only the gene-corrected SCID-X1 iPSCs

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Brief Report

Lymphoid Regeneration from Gene-Corrected SCID-X1 Subject-Derived iPSCs

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SUMMARY

X-linked Severe Combined Immunodeficiency (SCID-X1) is a genetic disease that leaves newborns at high risk of serious infection and a predicted life span of less than 1 year in the absence of a matched bone marrow donor. The disease pathogenesis is due to mutations in the gene encoding the Interleukin-2 receptor gamma chain (IL-2Rγ), leading to a lack of functional lymphocytes. With the leukemogenic concerns of viral gene therapy there is a need to explore alternative therapeutic options. We have utilized induced pluripotent stem cell (iPSC) technology and genome editing mediated by TALENs to generate isogenic subject-specific mutant and gene-corrected iPSC lines. While the subject-derived mutant iPSCs have the capacity to generate hematopoietic precursors and myeloid cells, only wild-type and gene-corrected iPSCs can additionally generate mature NK cells and T cell precursors expressing the correctly spliced IL-2Rγ. This study highlights the potential for the development of autologous cell therapy for SCID-X1 subjects.

Primary immunodeficiencies (PIDs) constitute a large and heterogeneous group of rare heritable disorders, including X-linked Severe Combined Immunodeficiency (SCID-X1), Adenosine deaminase deficiency-SCID (ADA-SCID), and Wiskott-Aldrich syndrome (WAS), that result in an underdeveloped and/or functionally compromised immune system. Allogeneic hematopoietic stem cell transplant (HSCT) from a matched donor confers significant therapeutic benefit with over 90% success rates; however, the scarcity of HLA-matched donors makes this approach less universally viable (Mukherjee and Thrasher, 2013). Autologous transplantation of hematopoietic stem cells (HSCs) genetically corrected by targeted genome editing has been shown to be efficacious in proof-of-concept studies (Genovese et al., 2014) and even clinically effective upon lentiviral gene therapy for subjects with metachromatic leukodystrophy (Aiuti et al., 2013) and WAS (Biffi et al., 2013). However, these autologous HSCT-based approaches are limited by imperfect methodologies for the culture and expansion of HSCs ex vivo.

SCID-X1 is an inherited disorder affecting the immune system. It is caused by mutations in the common receptor gamma chain, a subunit of several cytokine receptors, including interleukin-2, -4, -7, -9, -15 and -21, that commonly leads to a lack of T cells, functional B cells, and Natural Killer (NK) cells, leaving these subjects severely immune compromised. SCID-X1 affects males with an estimated incidence of 1:50,000 newborns and these individuals are at high risk of infection due to defective generation of early lymphoid progenitors. Without treatment, life expectancy is less than 1 year with a poor quality of life. A proven cure is transplantation of HLA-matched bone marrow; however, such subject-matched donors are relatively limited in supply.

Gene therapy trials on SCID-X1 subjects were initiated in 1999 and promising initial results emerged showing T, B, and NK cell function comparable to that in age-matched controls (Cavazzana-Calvo et al., 2000). Unfortunately, after the development of T cell acute lymphoblastic leukemia (T-ALL) as a consequence of vector-mediated genotoxicity, the trials were prematurely halted in 2003 and a new therapeutic approach is still sought (Hacein-Bey-Abina et al., 2003, 2008).

Transcription activator-like effectors (TALEs) from the plant pathogen *Xanthomonas* are sequence-specific DNA-binding proteins (Bogdanove et al., 2010; Kay and Bonas, 2009; Kay et al., 2007; Römer et al., 2007) that can be engineered to bind any desired target sequence (Boch et al., 2009; Moscou and Bogdanove, 2009). A pair of TALE nucleases (TALENs) can be used to generate a double-strand break (DSB) at a specific genomic locus and consequently to mediate homologous recombination (HR) (Bedell et al., 2012). TALENs have been successfully employed to mediate site-specific genome modification by HR in human pluripotent stem cells (Hockemeyer et al.,

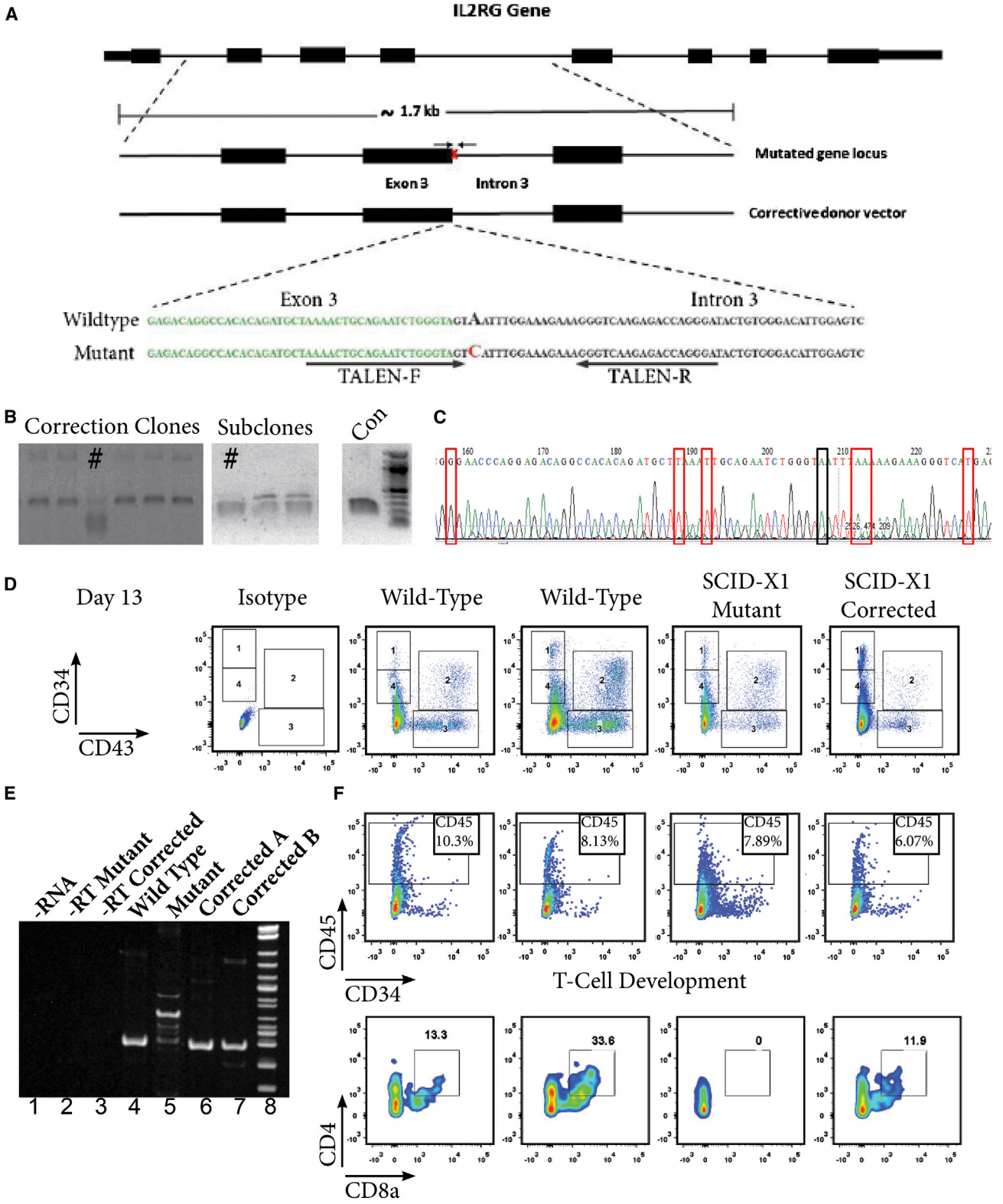

Figure 1. TALEN-Mediated Gene Correction and Lymphoid Differentiation of SCID-X1 iPSCs

(A) Schematic representation of the *IL-2Rγ* gene, the endogenous target locus of our subject-specific mutation, and the corrective donor vector used. The sequence of the subject-specific *IL-2Rγ* splice-site target mutation and the corrective donor vector sequence are also shown below. The point mutation causing

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2011), which offers the potential of an alternative gene/stem cell therapeutic approach.

NK cells are a key component of innate immunity and central to the host immune defense against pathogens and tumors (Biron et al., 1999; Vivier et al., 2011). NK cells have been successfully differentiated from $CD34^+$ cord blood cells (Kao et al., 2007; Meek et al., 2010). Differentiation from pluripotent stem cells has proved somewhat harder to accomplish. Initial studies identified putative NK cells from the differentiation of hESCs and iPSCs; however, these cells were characterized based solely on their expression of CD56 and the characterization lacked analysis of the functional receptors expressed on mature NK cells (Tabatabaei-Zavareh et al., 2007). As NK cells mature they start to express both activating and inhibitory receptors that regulate NK cell activity. Killer cell Ig-like receptors (KIRs) and CD94/NKG2 heterodimers are two major receptor types that interact with MHC on target cells. For iPSCs, however, the yield is considerably lower than for ESCs (Ni et al., 2011).

In the current study we were able to generate provirus-free iPSC lines from a SCID-X1 subject, correct the genetic defect utilizing TALENs, and differentiate these cells in vitro to NK cells expressing mature NK cell markers. Notably, while all tested lines were capable of generating myeloid and endothelial cells, only the wild-type and gene-corrected lines could differentiate into NK cells and demonstrated the presence of a correctly spliced IL-2Rγ. This is the first evidence of genomic correction of SCID-X1 subject iPSCs resulting in the regeneration of mature lymphoid cells in vitro and holds great promise for the development of novel therapeutic approaches for this incurable and terminal disease.

Eight iPSC lines were derived from bone marrow multipotent stem cells (BM-MSCs) from an infant with SCID-X1 using a Cre-excisable lentiviral vector containing six reprogramming factors (Firth et al., 2014). Control iPSCs were also generated from cord-blood-derived endothelial cells and dermal fibroblasts.

The donor subject harbored a novel splice-site mutation, c.468+3A > C, of the *IL-2Rγ*. This specific mutation results in a lack of functional NK cells and T cells (Ginn et al., 2004). The mutation is an A to C substitution in the third base pair of intron 3 of the *IL-2Rγ* gene, leading to aberrant splicing of the IL-2Rγ transcript. TALEN pairs were designed to target genomic sequences proximal to the described mutation (Figure 1A) and their functional activity at the desired target locus was validated (Figure S1A). The target SCID-X1 mutation was corrected by co-nucleofection of these TALENs in combination with a donor plasmid containing the corrective DNA sequence (Figure 1A). Corrected clones identified upon screening are shown in Figure 1B. Correction of the *IL-2Rγ* gene in each clone was verified by sequencing an integration-specific PCR product of the target genomic DNA, where correction of the target mutation at the endogenous chromosomal locus was detected along with the presence of silent mutations introduced in the corrective sequence (Figure 1C). Integration of the corrective *IL-2Rγ* sequence at its desired endogenous chromosomal locus was achieved at an overall efficiency of 2.6% without selection. The presence of the desired genetic correction and introduced silent mutations was confirmed by whole-exome sequencing of the corrected and parental iPSC lines, which also verified the lack of significant off-target effects in any coding sequence due to gene correction with TALENs (Figure S1).

The interleukin signaling affected by mutations in the common gamma chain should only have a significant impact on lymphoid differentiation and not on other hematopoietic lineages. As expected, each iPSC line was capable of proficiently generating $CD34^+$ hematopoietic progenitor cells and cells with cell surface markers of a hematopoietic stem cell—$CD90^+$ $CD34^+$, $CD43^+$, and $CD38^-$—which acquired CD45 expression upon commitment to the myeloid lineage (Figures 1D and S2A). The mutant and corrected cell lines tested showed a similar capacity for generating mature myeloid colony forming units (CFUs) in a methylcellulose-based maturation assay (Figure S2B). After 14 days of myeloid maturation, each line was equally capable of generating $CD45^+$ $CD14^+$ monocytes and $CD45^+$ Glycophorin A^+ erythroid progenitors (Figure S2C).

$CD34^+$ progenitor cells derived from embryoid bodies (EBs) were sorted at day 8 of differentiation and plated on OP9 cells overexpressing DL-1. After 14 days of differentiation on OP9-DL-1 in the presence of IL-7, we were able to generate $CD45^+$ cells in the floating fraction that also co-expressed CD4 and CD8a. The mutant SCID-X1 line only generated $CD45^+$ cells; no significant population of $CD4^+/CD8a^+$ cells was observed (Figure 1F). We were able to see the rescue of the aberrant splicing of the IL-2Rγ transcript in $CD45^+$ T cell precursors derived from the gene-corrected iPSC lines as compared to the subject iPSC-derived mutant $CD45^+$ cells (Figure 1E, compare lane 5 with 4, 6, and 7).

To determine whether lymphoid potential was restored in the corrected iPSC line, we first produced HSCs from day 15 EB cultures from each of the indicated GFP-transduced iPSC lines, purified them based on CD34 expression, and transferred these to AFT024 layer cultures (Ni et al., 2011). Hematopoietic precursors isolated from SCID-X1 mutant iPSCs did not differ markedly from those of the various controls, generating similar percentages of

the alteration of this exon/intron consensus splice site is indicated in red. Exon 3 sequence immediately preceding the splice site is denoted by green, with bases in bold. TALEN binding sites are indicated by black arrows.

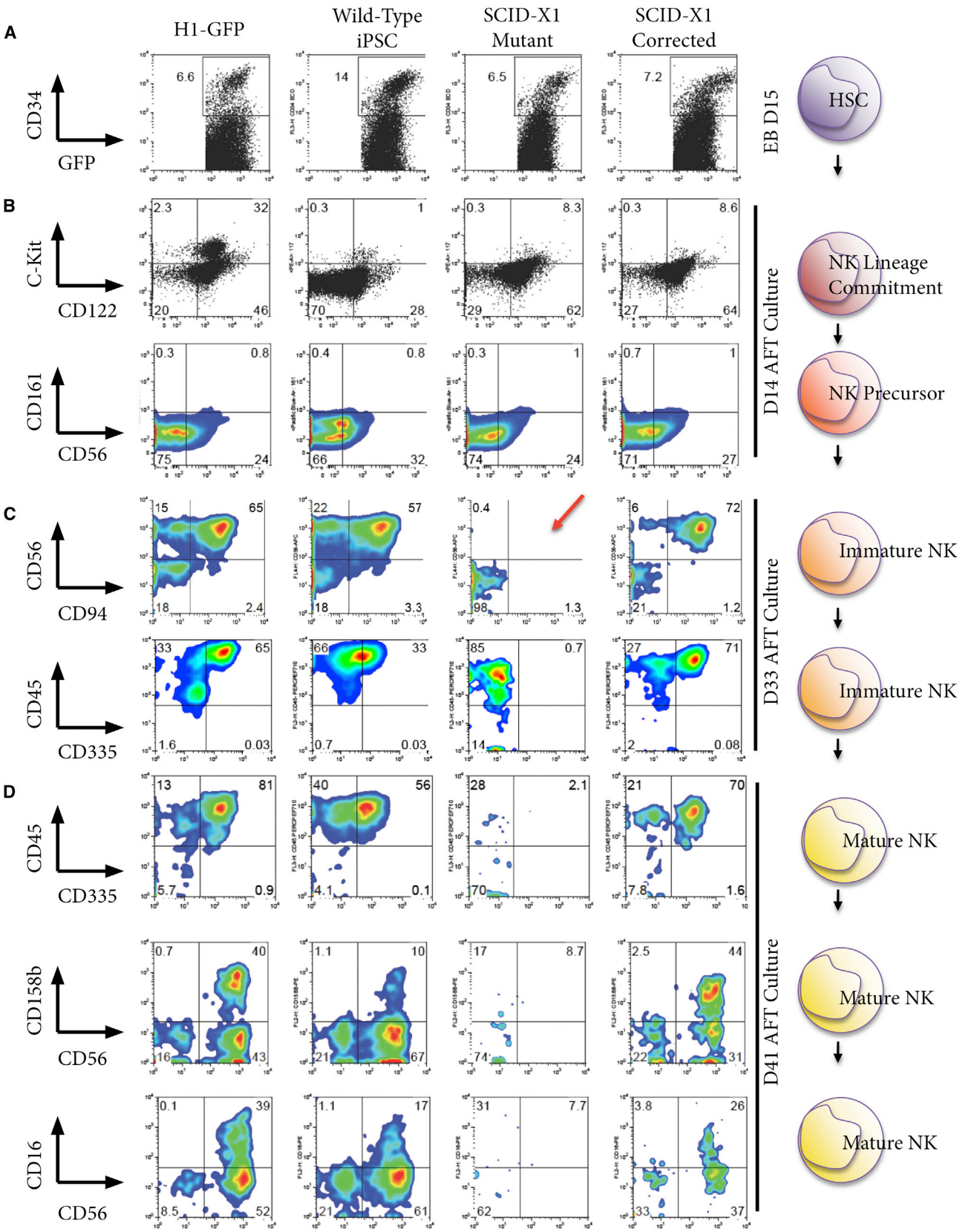
(B) Identification and isolation of corrected iPSCs through single-cell clonal amplification and screening of the PCR products with an XmaI restriction digest that is specific to the correction event. Corrected clones and subclones are identified by (#).

(C) Chromatogram of the corrected iPSC clone indicated in Figure 1B, as verified by sequencing. The red boxes indicate each of the silent mutations that were introduced to abolish TALEN activity on the corrective or corrected DNA sequences. Black box indicates the corrected disease-causing base.

(D) Comparative analysis of FACS data from wild-type, SCID-X1 mutant, and SCID-X1 corrected iPSCs. Data show CD34 and CD43 expression at day 13 of differentiation. Isotype controls are included in the left panel.

(E) RT-PCR analysis of RNA extracted from T cell precursors in the floating fraction generated from wild-type, subject-derived SCID-X1 mutant iPSCs, or SCID-X1 corrected iPSC clones.

(F) FACS analysis of the floating fraction of cells co-cultured on OP9-DL feeders from two independent wild-type, SCID-X1 mutant, and SCID-X1 gene-corrected iPSC lines. $CD45^+$ and $CD45^+/CD4^+/CD8a^+$ populations are indicated.



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$CD34^+$ in EB culture by day 15 (Figure 2A). HSCs derived from all lines generated cells that could progress to early immature NK cells as indicated by expression of CD122 and extremely low level expression of CD117 (c-kit), CD161, and CD56 (Figure 2B). Thus, in vitro, this subject's defect in common γ-chain signaling does not prevent entry and early progression within the NK lineage.

By day 20, however, differences become evident when CD94 and CD56 emerge in control and corrected SCID-X1 cultures but are absent in SCID-X1 cells (data not shown). The difference becomes much more pronounced as the cells progress; by day 33 of culture there is a clear defect in differentiation of the SCID-X1 cells. This suggests that these cells cannot pass into the early immature NK developmental stage as defined by expression of these two markers (Figure 2C). Similarly, while SCID-X1-derived cells in AFT culture are clearly hematopoietic, because they strongly express CD45, they are negative for CD335, a marker uniquely expressed on NK cells (Figure 2C). To rule out the possibility that the SCID-X1 cells were slower in developing than the wild-type or gene-corrected cells, we continued a long-term differentiation to day 41, at which point very few $CD45^+$ SCID-X1 cells remain, and no evidence for their further development was observed. In contrast, the gene-corrected and control lines progressed to mature NK status, expressing both inhibitory and activating receptors (KIR/CD158b and CD16, respectively) found on mature NK cells (Figure 2D). These cells continued to express CD335, which was never observed in the mutant cells. This demonstrates that correction of the IL-2Rγ mutation in SCID-X1 iPSCs results in restored developmental progression along the NK lineage to cells expressing multiple mature NK cell markers.

In this report, we have demonstrated that the precise correction of a novel splice-site mutation of the *IL-2Rγ* gene at its endogenous genomic locus in subject-derived iPSCs results in the correction of the splicing defect of the IL-2Rγ transcript and thus rescues the ability of iPSCs to differentiate into NK cells. It is the first report, to our knowledge, to describe the derivation of iPSCs from a SCID-X1 subject and the functional gene correction of a point mutation using genome editing technology using a selection-free approach. Upon gene correction, we generated mature NK cells and T cell precursors that were otherwise abnormal or missing in the subject.

Improved viral approaches for gene therapy have been developed since the original SCID-X1 clinical trials (Hacein-Bey-Abina et al., 2002). Lentiviral gene therapy using autologous HSCs has proven initially efficacious in the treatment of WAS (Aiuti et al., 2013; Verma, 2013) and metachromatic leukodystrophy (Biffi et al., 2013) subjects. An ongoing clinical trial using a self-inactivating γ-retrovirus vector to transduce autologous $CD34^+$ HSCs with the IL-2Rγ gene for transplantation in nine SCID-X1 subjects has also shown early clinical benefits (Hacein-Bey-Abina et al., 2014); these studies require long-term follow-up to conclusively determine the safety of this approach. Leukemogenic outcomes were observed in the original trial as late as 68 months after gene therapy. All viral gene therapy vectors involve integration into the host genome and consequently bear the risk of insertional mutagenesis on a subject-to-subject basis.

Direct genome editing of subject-derived $CD34^+$ HSCs using zinc finger nucleases (ZFNs) was shown in a recent proof-of-concept study (Genovese et al., 2014). While this is an attractive alternative to viral gene therapy, these approaches are dependent on the hitherto elusive capability to efficiently culture and expand long-term repopulating HSCs ex vivo. In addition, HSCT-based approaches involve myeloablative pre-transplant conditioning, which poses a particularly high level of risk for these SCID-X1 subjects, given their very young age and highly immune-compromised state. An iPSC-based approach provides an unlimited source of subject-derived, corrected cells from which immune cells can be derived continuously for transfusion, and this approach could serve as a complementary approach to treat subjects.

Adoptive transfer of NK cells is already being used to treat a number of malignancies (Geller et al., 2011; Ruggeri et al., 2002), thus creating an unlimited source of subject-specific NK cells, which could provide significant clinical benefit in the treatment and management of SCID-X1 and similar diseases. Further improvements and scaling up of our current protocols will be necessary to obtain sufficient cells for use in a clinical context. In summary, we have described here the precise genetic correction of a SCID-X1 subject-derived iPSC line, and importantly, we provide evidence for recovery of lymphoid differentiation from these isogenic gene-corrected iPSCs, creating an ideal platform for improved modeling and therapy of SCID-X1 and similar PIDs.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Supplemental Experimental Procedures and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2015.02.005.

AUTHOR CONTRIBUTIONS

The author contributions in this manuscript were as follows: A.L.F. and T.M., study conception and design, acquisition of data, analysis and interpretation of data, and drafting of the manuscript; D.S.A and Z.G., acquisition of data, analysis and interpretation of data, and drafting of the manuscript; S.J.Q., W.B.G., O.S., L.S.A., and A.R.B., acquisition of data; E.K., analysis and interpretation of data; I.E.A., provision of materials; and J.Z. and I.M.V., study conception and design and drafting of the manuscript.

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Figure 2. Generation of Mature NK Cells

FACS analysis of dissociated cells from H1-Embryonic Stem Cells (ESC), wild-type iPSCs, SCID-X1 mutant iPSCs, and SCID-X1 gene-corrected iPSCs for the antigens indicated on the left at the following time points: (A) embryoid bodies (EB) at day 15; (B) NK lineage committed cells/NK precursors at day 14 of AFT culture; and (C) immature NK cells at day 33 of AFT culture. The red arrow highlights the lack of CD56/$CD94^+$ cells in the mutant iPSC and (D) mature NK cells at day 41 of AFT culture. Cell percentages are indicated at the corner of each gate.

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